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14. ABSTRACT Neoplastic meningitis is a severe complication of breast cancer for which there is no curative treatment at present. The project aims to develop a novel, safe and efficient therapy for neoplastic meningitis – HSV-1 oncolysis therapy. For the first year of the award period we have propagated the HSV-1 replication conditional mutant (hrR3) with burst assays and generated three different virus preparations to establish viral titers of 1×10^7 , 1×10^8 and 1×10^9 pfu/ml. The virus titers were determined by plaque assays performed on vero mono-layers. These virus titers will be used to study fractionated viruses particles. In addition we have commenced on additional work that will help study the disease progression by molecular imaging. In anticipation of establishing a mouse model of meningeal metastasis we have developed stable breast cancer cell lines that express bioluminescence and fluorescence markers for in vivo molecular imaging. Three cell lines (human breast cancer cell lines SkBr3 and MDA-231, and the mouse breast cancer 4T1) were transfected to express renilla luciferase (RLuc) and mCherry, both driven by the CMV promoter. Clones that were resistant to puromycin were selected to establish stable cell lines. These stable cell lines have been tested for (i) the replication efficiency HSV-1, and (ii) their sensitivity to the HSV-1 oncolysis with in vitro bioluminescence. Based on these preliminary data we anticipate proposing the use of one of the cell lines to establish a mouse model of meningeal metastases. We will in due course approach DOD for approval of the animal protocol.					
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Introduction:

Neoplastic meningitis is a devastating complication of breast cancer and other solid tumors. Approximately 5-8% of patients suffer from this complication with nearly 5% of the patients presenting metastatic meningitis on the first relapse, and 20% on the second. Neoplastic meningitis develops in the space enveloped between the brain and the external meningeal sheath (dura mater) that fully surrounds the brain. Seeding of the leptomeninges, the arachnoid and pia mater, by cancer cells causes neoplastic meningitis (Chamberlain 05). Treatment options at present such as craniospinal irradiation, systemic chemotherapy, and local chemotherapy via lumbar puncture or intraventricular (Ommaya) reservoir is largely palliative. As the leptomeningeal space is well isolated from the rest of the body by the blood-brain barrier (BBB) on one side, and by the blood-CSF barrier on the other, systemic chemotherapy is ineffective as the therapy does not reach the meninges where the cancer cells reside. A drug that would stay in the CSF for a sufficiently long period of time (several hours or days) and have access to the cancer cells would efficiently terminate the meningeal cancer spread and greatly improve the outcome of chemotherapy.

Our strategy is aimed at testing the hypothesis that an oncolytic virus injected to CSF will effectively suppress and/or eradicate the meningeal cancer spread. Viral oncolysis, the destruction of cancer cells by replicating viruses, is under clinical investigation for cancer therapy. Hence, the goal of the proposed study is to develop novel, safe and efficient therapy for neoplastic meningitis – that of HSV-1 oncolysis. The viral genome is genetically engineered to generate replication conditional mutants that replicate preferentially in cancer cells rather than in normal cells (Kuruppu et al 05).

We present here the work that has been accomplished over the first year of the grant period based according to the SOW. We also present some preliminary work that would be relevant for establishing a mouse model of meningeal metastases. This work is beneficial as a platform for which the rat model proposed in the grant can be accomplished to maximal heights.

Body:

SOW performed:

Based on the statement of work we have maintained and prepared the virus, and estimated the virus titers for the studies in the rat model of neoplastic meningitis.

1. Prepare virus and burst assays

The HSV-1 mutant (hrR3) that we plan to use in our experiments was propagated in vitro in vero (African green monkey kidney cells) cells over time. The burst assays were performed to propagate the virus in mass cultures in 150cm tissue culture flasks. The virus was prepared by several rounds of centrifugation at different centrifugal forces- first to pellet the cells and ultimately pellet the virus. Briefly, the collected virus and cells are centrifuged at 3000rpm to pellet cells and cell debris. The supernatant is again centrifuged to pellet any residual debris. The resulting supernatant from the second centrifugation is subjected to high speed centrifugation (17,000rpm) to pellet the virus. This pellet is resuspended to obtain the desired viral titer that will be determined by plaque assay (Kuruppu et al).

2. Perform plaque assays for estimation of virus titer

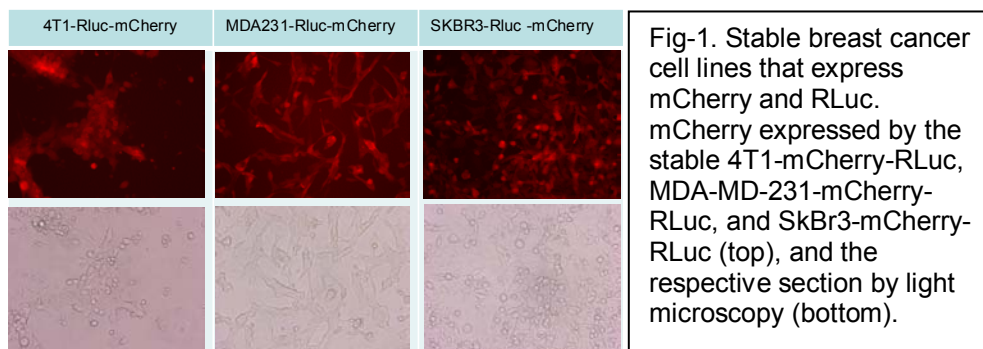
The titer of virus (hrR3) was performed in vero cell monolayer cultures in triplicates to determine the titer of the virus (Nakamura et al). Vero cell monolayers once they reach 80% confluency are infected with the newly prepared virus at a multiplicity of infection (MOI) of 1. The infected vero monolayers are overlaid with an agarose layer with serum and plaques are allowed to form over 5 to 7 days. The plaques are counted and stained further for blue color as hrR3 expresses the β -galactosidase gene. We prepared hrR3 at titers of 1×10^7 , 1×10^8 and 1×10^9 pfu/ml. Each of these viral titers were prepared in individual burst assays. Each viral titer is maintained in 100ul aliquots (with 10 vials of 100ul) and stored in liquid nitrogen until used for the scheduled experiments.

Additional work performed:

We have developed cell lines that express bioluminescent and fluorescent markers that we plan to use in an animal model in the following year. These cell lines were also tested for the replication efficiency of the HSV-1 (HSV-Luc) and their sensitivity to HSV-1 (HSV-Luc) oncolysis in vitro with dual bioluminescence.

3. Develop cell lines for future use in bioluminescent and fluorescent imaging

The human breast cancer cell line (SkBr3) that we had proposed to use in our rat model of neoplastic meningitis was engineered to express the bioluminescent renilla luciferase gene and the fluorescence mCherry gene by stable transfection. As both these molecular imaging markers are driven by the CMV promoter, they are expressed simultaneously. Following transfection, clones that were resistant to puromycin were selected and each clone was propagated into a stable cell line that expresses RLuc and mCherry. Two other breast cancer cell lines (the human MDA-231, and the mouse 4T1) cell lines were also transfected to generate stable cell lines expressing RLuc and mCherry.



4. Perform preliminary confirmation studies on the stable cell lines

4 (a). Determine the efficiency of HSV-1 replication in each of the cell lines.

We tested two of the cell lines (4T1 and SkBr3) for their bioluminescence expression and their affinity to enable virus replication. Each cell line was plated in 24 well dishes and infected with different HSV-1 virus titers ranging from 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 and 1×10^7 pfu/ml. We used the HSV-1 virus (HSV-Luc) that expresses the Firefly luciferase (FLuc). The vero cells in which the HSV-1 virus is propagated was used as a control for virus replication efficiency in each of the cell lines. One day after infecting the cells with virus, the expression of virus in the cells was determined with bioluminescence with luciferin. The virus replicates robustly in the vero cells. In comparison, its replication efficiency in SkBr3 cells is greater than in 4T-1 cells.

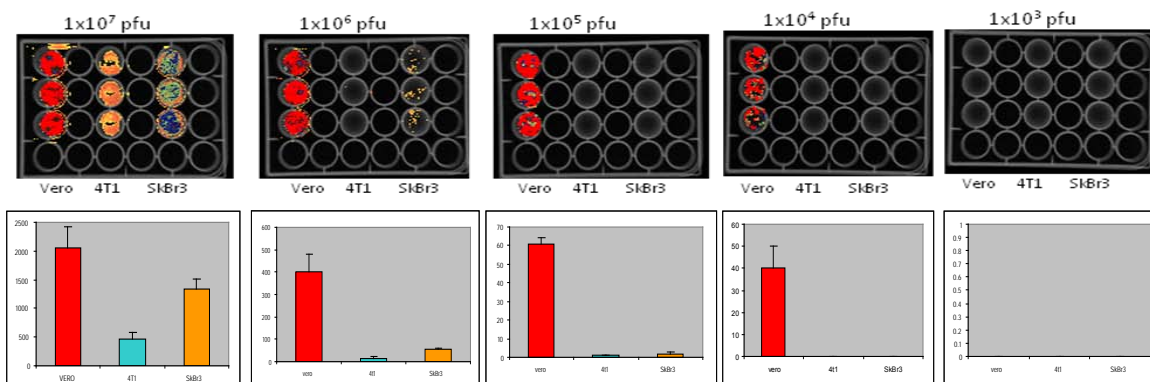


Fig-2. HSV-1 replication efficiency in breast cancer cell lines. Vero cells, 4T1 and SkBr3 cells plated to 80% confluency were infected with different virus titers (1×10^3 pfu to 1×10^7 pfu). Virus replication in the cell lines was studied by bioluminescence for Fluc expressed by the HSV-Luc with luciferin.

4 (b). Determine the sensitivity of the cell lines to HSV-1 oncolysis.

All three cell lines were tested for their sensitivity to virus. Each cell line was plated in triplicate in 24 well plates and exposed to different titers of virus (HSV-Luc) ranging from 1×10^5 , 1×10^6 and 1×10^7 pfu/ml. One day post infection of the cells the activity of the cells were determined by their RLuc expression with coelenterazine. The cytopathic effect of the virus was compared to a non-virus treated control. It appears that the MDA-231 cells are more susceptible to the virus than the other two cell lines.

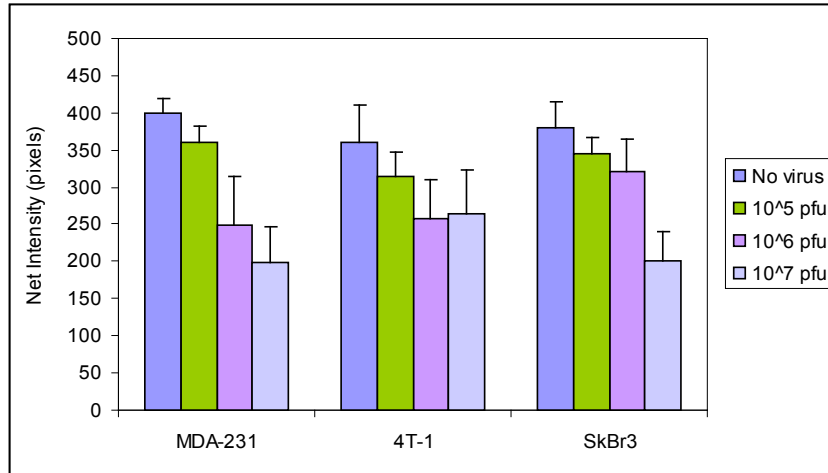


Fig-3. Sensitivity of the stable breast cancer cell lines to HSV-1 oncolysis was determined by bioluminescence in vitro. Confluent cells were infected with virus titers ranging from 1×10^5 , 1×10^6 and 1×10^7 pfu/ml. Cell destruction was determined for each of the cell lines by RLuc expression with coelenterazine. The data were compared to the cells that did not get virus with coelenterazine.

5. Prepare for pilot studies to generate a mouse model of meningeal metastases that can be treated with HSV-1 oncolysis

We are in the process of getting approval for a mouse model for experimental meningeal metastases. Once this approval from the MGH SRAC is granted we will approach the DOD to get approval to use mice in our grant protocol prior to commencing any of the experiments on the mouse model of meningeal metastases. Thus far we have approval from DOD for the use of rats only.

Our plan is to generate a mouse model by intra-ventricular injection of cells (2000 cells in 10ul). With this approach the cells will migrate towards the cerebrospinal fluid. We will monitor the tumor cell metastases and their establishment by imaging for RLuc that the cells express (with coelenterazine) over time. Once the model is established we plan to test the efficacy of the HSV-1 virus. The mouse model will serve as a pre-study model optimization and characterization with which we can launch on our rat experimental model. The mouse model will serve as a valuable tool with which tumor cell growth can be monitored by non-invasive bioluminescence imaging and quantified.

Key Research Accomplishments:

For the stipulated grant period we have performed the following preliminary work towards generating the proposed data and for future experiments that we plan to accomplish for the grant.

1. Prepare virus and burst assays
2. Perform plaque assays for estimation of virus titer
3. Develop cell lines for future use in bioluminescent assays
4. Perform preliminary confirmation studies on the cell lines
5. Prepare for pilot studies towards generating a mouse model of meningeal metastases that can be treated with HSV-1 oncolysis

Reportable Outcomes:

We have propagated virus and established their titer for future studies on the rat model with neoplastic meningitis. In addition, we have developed stable cell lines that express bioluminescent and fluorescence markers (RLuc and mCherry respectively) to enable the cells to be imaged in vivo. Using the stable cells that we have generated we propose to develop a mouse model of meningeal metastases that can be used to study the therapeutic efficacy of viral oncolysis. This will be initially achieved with bioluminescence to be followed with PET imaging.

Conclusion

We have performed the work that was relevant for the virus fractionation for year one. In addition we have generated new data for a new arm of the project –to develop a mouse model of meningeal metastases. This model will better help study the disease process on a continual basis by non-invasive monitoring of tumor progression with bioluminescence. Following establishment of the mouse model we will test the efficacy of the oncolytic HSV-1 with the use of the HSV-1 virus that expresses the firefly luciferase gene, and hence monitor the sites and replication of the virus with in vivo bioluminescence imaging. If successful, it will be incorporated into the protocol. Prior to commencing any work on the mouse model we will seek approval from the DOD to incorporate the mouse protocol in the near few months. This will provide a platform for PET imaging that we have proposed to achieve in our rat model of neoplastic metastases.

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